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ELECTROPHORETIC AND DIFFUSION STUDIES ON GLUTEN

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FACULTY OF AGRICULTURE

The undersigned hereby certify that they have read and recommend to the Committee on Graduate Studies for acceptance a thesis on "Electrophoretic and diffusion studies on gluten", submitted by J. R. Colvin, B.S.A., in partial fulfilment of the requirements for the degree of Master of Science.

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ELECTROPHORETIC AND DIFFUSION STUDIES ON GLUTEN

J. R. Colvin

Department of Plant Science

A THESIS

submitted to the University of Alberta

in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE

Edmonton, Alberta

April, 1948

C. V. Cotrio
Department of Plant Sciences

A Thesis

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ELECTROPHORETIC AND DIFFUSION STUDIES ON GLUTEN

J. R. Colvin

INTRODUCTION

The properties of wheat proteins, and particularly of gluten, have occupied the attention of cereal chemists for over two hundred years. Becarri (6) was the first to recognize the resemblance between the characteristics of a fraction of wheat flour and those of animal flesh. Following this fundamental observation, Taddei, Einhof, De Saussure, Leibig and Ritthausen attempted, by extensive fractionation and analytic techniques, to characterize the product obtained by washing starch from wheat flour. Bailey (5, Chap.1) has given a good review of the work done during this period. Ritthausen (5), on the basis of observations of differences in solubility and chemical composition, regarded the proteins of wheat as belonging to five groups:- 1. Gluten casein. 2. Gluten fibrin. 3. Plant gelatin. 4. Mucedin. 5. Albumin. Owing to the crudeness of the chemical techniques available at the time his classification must, however, be regarded as of historical interest only.

Ritthausen's work was amplified by later investigators but the next great monograph issued on the subject was that written by Osborne in 1907 (40). Since Osborne's report was the foundation for the greater part of the work done on wheat proteins in the first quarter of the twentieth century, his division of these substances will be listed in some detail. He based his classification on solubility and composition differences but used greatly improved techniques. Osborne believed that the nitrogenous substance of the wheat berry was a mixture of the following distinct component proteins.

(a) Albumin (leucosin) - Present in relatively small amounts (.3% to .4% of the kernel) this protein was soluble in water and 10% NaCl but insoluble in saturated $(NH_4)_2SO_4$. It was coaguable by heat.

(b) Globulin - This was soluble in 10% NaCl but insoluble in water. It was insoluble in saturated $(NH_4)_2SO_4$.

(c) Gliadin - This component, which, with the glutenin, made up much the greater part of wheat protein, was slightly soluble in water, soluble in dilute ethyl alcohol as well as other alcohols but insoluble in absolute ethyl alcohol. It was dispersed in dilute acids or alkalis but precipitated in neutral solutions of salts.

(d) Glutenin - This corresponded to the plant casein of Ritthausen. It was insoluble in water and cold alcohol but slightly soluble in hot alcohol. It dispersed easily in dilute acids and alkalis.

(e) Proteose - The properties of this fraction, which was present in very small amounts, were acknowledged to be uncertain.

Osborne's concept of wheat protein as a mixture of chemically distinct molecules dominated the thinking of cereal chemists for a quarter of a century. Since the gliadin and glutenin were found to make up by far the greater fraction of wheat gluten, it was naturally assumed that they were jointly responsible for most of its properties and an enormous amount of effort was spent on the isolation and characterization of each of these "proteins." In particular, the ratio in which they were supposed to occur in wheat was thought to be correlated with the physical properties of the gluten and with the baking quality of flour. Much time was spent on the estimation of the optimum proportions. This work has also been amply reviewed by Bailey (5, Chap.2).

Beginning about 1930 and continuing to the present, results have been published which are at variance with Osborne's concept. Cook (9) showed that there was no clear-cut distinction between the solubilities of gliadin and glutenin in 30% urea solution. This destroyed Osborne's original basis for separating the two "proteins," at least in-so-far as this solvent was concerned. A little later, Sinclair and Gortner (52) showed that in the more traditional solvents, alcohol and dilute salt solutions, the solubility of the "gliadin" and "glutenin" fractions was very largely a function of previous treatment and

(a) Increase - the properties of their creation which was known
in very small degree, were considered to be unneeded.

Gabarro's concept of waste product as a mixture of
chemically distinct molecules dominated the field of product
chemistry for a quarter of a century. Since one element was
plastic were found to make up by far the greater fraction of
waste glass. It was naturally assumed that they were jointly
responsible for most of the problems in the economic manner
of effort was spent on the isolation and characterization of
each of these "problems." In particular, one tried to assign
they were supposed to occur in water and showed to be
correlations with the chemical properties of the latter and with
the overall quality of flow. Much time was spent on the
refinement of the optimum proportions. This work was also based
mostly reviewed by Kelly [3, chapter].

Beginning around 1950 and continuing to the present,
results have been published which are at variance with Gabarro's
concept. Work [3] showed that there is always dissolved
between the solubilities of glycin and glycerin in the same
solution. This observation destroyed Gabarro's original basis for
the idea of "pollution," of large importance to this science was
removed. A little later, Kimball and Turner [2] showed
that in the more traditional schemes, almost any mixture had
solutions. The solubility of the "ideal" was "of limit"
Precision was very large. A function of previous treatment and

suggested that the various peptization behaviours were a reflection of the physical characteristics of gluten rather than of chemical characteristics. This too was supported by work of Cook (9) who was unable to find differences in the composition and chemical properties of various preparations of gliadin commensurate with differences in physical properties. Blish and Sandstedt (7), Herd (18) and Haugaard and Johnson (17) using extraction techniques threw serious doubt on the chemical distinguishability of "glutenin" while early diffusion and ultracentrifuge work (23, 24, 26) on gliadin showed that this gluten fraction was definitely inhomogeneous as far as mass characteristics are concerned. All this work suggested that the whole concept of a simple mixture of two distinct proteins required revision.

The hypotheses as to the nature of gluten, stemming from the above results, were two. One group, led by Sandstedt and Blish (46) believed gluten to be made up of three, main, dissociable, component systems, each of which was capable of splitting into lesser complexes, following Sørensen's theory of soluble proteins (54). On the other hand, McCalla and associates on the strength of reversible fractionation results (36, 55) were led to the view that gluten was a single protein complex consisting of an association of molecules which varied progressively and continuously in physical and chemical properties. It is obvious that the two views are the same if the number of main dissociable component systems of Sandstedt and Blish becomes quite large.

In 1942, McCalla and Gralen published the results of an extensive study on gluten in sodium salicylate solutions using ultracentrifugal and diffusion methods (37). These results proved that in sodium salicylate gluten existed in two forms, molecularly dispersed and aggregated. There was, however, progressive continuous variation in the physical properties and dimensions of both the molecules and the aggregates. This proved conclusively that "gliadin" and "glutenin" did not exist as distinct entities and also gave strong support to the view that gluten is a reversible protein complex, as suggested.

The study mentioned above proved the heterogeneity of gluten in sodium salicylate solutions as far as mass characteristics are concerned, but no corresponding study of the electrical characteristics of gluten in sodium salicylate has been made. Moreover, only a very limited amount of work has been done on gliadin, gluten or related proteins in other solvents. Wood and Hardy (59) had shown that the physical properties of a gluten ball were a function of the electrical charge on the gluten micelles which in turn depended on the hydrogen ion concentration of the surrounding medium. They proved that the gluten micelles had a positive charge in acid medium and a negative charge in alkali, as was to be expected. Moreover, they could find no differences in the behaviour of gluten and of a sample of gliadin. On the basis of these results they concluded that the physical state of gluten was largely determined by the potential difference between the protein and the

dispersing fluid. Kemp and Rideal (22) and Kemp (21) have used the microelectrophoretic technique with gliadin adsorbed on quartz to estimate the isoelectric point of gliadin and to compare the surface charge densities of various flours. They found an isoelectric point of approximately 5.00 for gliadin but this value was inversely dependent on ionic strength. Despite extensive data on the adsorption of gliadin on quartz, few data were given on the surface electrical properties of gliadin itself.

The only study of gliadin using the Tiselius electrophoretic technique was that of Schwert, Putnam and Briggs (49). Dispersing gliadin in acetate buffers they found that it was markedly heterogeneous. Two main fractions of widely different mobilities and isoelectric points were identified but these two fractions themselves could be subdivided into components. Because of this heterogeneity and also because of a strong asymmetry between patterns obtained in the two legs of the cell these workers concluded that gliadin was a mixture of components with widely different surface charges interacting in a non-ionic manner.

While the present study was in progress Scallet (47) published a report of a study on zein in aqueous alcohol using the Tiselius electrophoretic apparatus. As the result of multiple fractionations followed by electrophoretic analysis, he concluded that zein was a typical association-dissociation system as described by Sørensen (54).

Since electrophoretic data naturally supplement ultracentrifugal and diffusion data (1, p.131) this study was undertaken. It seemed logical to suppose that the known variation in the chemical composition of gluten fractions (36) would be reflected in their electrical behaviour and information on such variability would lead to a better understanding of the mechanics of the gluten complex. Specifically, it was hoped to determine the number of components in gluten and their electrophoretic mobilities. Later, this objective was broadened to include the charge density of the molecularly dispersed gluten particle. These data, with existing ultracentrifugal and diffusion values, might then allow an estimate of the size and degree of hydration of the molecule (1, Chap.5).

MATERIALS AND METHODS

The gluten used throughout this study was obtained from an unbleached high quality flour milled from hard red spring wheat by one of the local mills. The ash and protein content of this flour were 0.45% and 14.2% respectively. When baked by a standard malt-phosphate-bromate formula, this flour gave high quality loaves of good volume and texture. The gluten itself was in every way typical of that from the best Canadian spring wheat, being firm, elastic and extensible.

The solutions of sodium salicylate used for dispersion were made up carefully by weight on a laboratory torsion balance from C.P. reagent grade chemicals. Unless otherwise stated, all solutions used were of 0.5 ionic strength (0.5 μ) which corresponds to slightly less than 8% sodium salicylate by weight. All preparations were checked before use against a standard solution by a conductivity apparatus.

Because McCalla and Gralén had found that gluten existed in two different forms in sodium salicylate, the electrophoretic properties of two series of gluten were investigated; i.e. molecularly dispersed gluten and whole gluten. Each series was represented by four samples prepared from the flour in as uniform a manner as possible and stored under the same conditions. The molecularly dispersed series was prepared as described by McCalla and Gralén for their C IV fraction except that the final dispersion was made in 0.5 μ solution rather than in 12% sodium salicylate. Each of the samples in the "whole gluten" series was prepared by washing the starch from 100 g. of ether-extracted flour with Dill and Alsberg's buffer (12) for ten minutes. The resulting gluten ball was immediately torn into small pieces and allowed to disperse in a liter of sodium salicylate solution for three days with occasional gentle shaking. The undispersed gluten was then removed by centrifuging and the opaque solution clarified by adding magnesium sulphate until the solution was .015% saturated with respect to this salt. After removal of the precipitate by

a second centrifugation, the solution was diluted to a protein content of from 0.6% to 0.8% and stored at 5°C. until used.

Electrophoretic mobilities and patterns were obtained at 2.0° ± 0.05 using a Tiselius apparatus manufactured by Klett Manufacturing Co. and described by Longsworth (30). Details of dialysis of sample, cell manipulation and instrument handling were essentially as given in papers by Longsworth, MacInnes and Svensson (28, 30, 33, 34, 57) except for the following modifications. It was found that the power supply using a #80 rectifier tube could not maintain a constant voltage at the relatively high currents needed (20 to 25 m.a.). Accordingly, B batteries in series were substituted as a source of potential. This arrangement held the voltage satisfactorily constant. Longsworth (29) has described the use of a cell which eliminates errors in mobility due to movement of the rubber sleeves on the original Tiselius apparatus. Since no Longsworth cell was available, we were forced to use a Tiselius cell. This necessity has been reflected in the lack of precision in the pH-mobility curves (see Fig. 5) and is the greatest single source of error in this study. It was accentuated by the low mobility of the gluten in the buffer.

Mobilities were calculated using the method of Longsworth and MacInnes (34) for locating the boundary and the formula $u = \frac{VK}{It}$. Since no microcomparator was available, the negatives were enlarged approximately four times and measurements were

made directly on these enlargements. By this method boundaries could be located within .01 cm. which, with a boundary travel of 1.5 cm., represents an error of 0.6%. This is well within the limits of error set by the source mentioned above.

Conductivities were measured using an Industrial instrument which on testing with a calibrated cell showed an accuracy of ±1.0%. This comparatively low order of accuracy had to be used since no better instrument was immediately available. The relative error due to this factor was probably reduced by the fact that the conductivity of the 0.5 μ solution was quite constant at the given temperature for different samples.

Hydrogen ion concentrations were measured with a Beckmann Model G pH meter to ±0.05 units. It will be noticed that no buffer salts were used in addition to the main electrolyte. This was because repeated preliminary tests showed that no changes in hydrogen ion concentration took place in the boundary area even after 10 hours of passing a 25 ma. current.

During the course of the study it was found necessary to determine the diffusion constant of molecularly dispersed gluten at 2.0° ± .05 in sodium salicylate. This was done by supporting a Neurath cell (38) in the thermostat of the electrophoresis apparatus at a position where the magnification was unity and using the formula $\frac{A^2}{4\pi t(H_m)^2} = D$ for calculation from the patterns obtained as by Rothen (45). With a polydisperse substance such as gluten, this formula yields only a mean value

for the diffusion constant but this was satisfactory for a first approximation. A test of the method on .1 M sucrose at 20° gave a mean diffusion constant from twelve determinations of (44.3 ± 0.9) X 10^{-7} moles/cm^2/sec. as compared with the value from International Critical Tables of (44. ± 2.) X 10^{-7} moles/cm^2/sec.

Viscosities were determined using an Ostwald viscosimeter calibrated against water at 2.0° ± 0.05. Techniques were as described by Findlay (13).

After several unsuccessful attempts to determine the charge on the gluten molecule by means of membrane potentials (2, 3) the moving boundary method as suggested by Longsworth (31) and Svensson (57) was modified for use with gluten in sodium salicylate. Since Longsworth had developed the theory in greater detail and checked it against other methods for egg albumin, his procedure was followed. Essentially, for a uni-univalent electrolyte, the application of this method depends on the determination of the relative mobilities of the protein, the buffer cation and the buffer anion, together with the change in refractive index per equivalent of buffer and the change in refractive index at the buffer-concentration boundary (Longsworth's nomenclature) per unit concentration of protein in the moving boundary. Then, by application of the formula taken from Longsworth (31),

$$n^{\beta} - n^{\gamma} = K_{AR}\left(\frac{r_B - r_R}{r_B}\,\frac{r_A}{r_A - r_R} - \frac{1}{2}\right) 10pe$$

where $n^{\beta} - n^{\gamma}$ = change in refractive index at buffer-conc. boundary,

K_{AR} = change in refractive index per equivalent of electrolyte,

r_B = relative mobility of protein

r_R = " " " anion

r_A = " " " cation

p = grams protein/100 ml.

e = charge in Faraday equivalents per gram of protein,

after substitution of the appropriate constants, it is possible to calculate e . If the molecular weight of the protein is known, this allows an estimate of the "valence" of the protein ion at the selected pH. Since most of these constants for sodium salicylate are not in the literature it was necessary to determine them.

The K_{AR} value for sodium salicylate at 2.0° was estimated by an Abbé refractometer used on ten samples of solution of progressively varying concentration. This value is accurate to at least 0.3%.

The absolute mobility of the protein ion can be found from the electrophoretic measurements but in order to find its relative mobility it was necessary to know the mobilities of the sodium and salicylate ions at 2.0° in 0.5 μ sodium salicylate. Such values are not given in the literature and the apparatus necessary for ascertaining them directly was not available.

Accordingly, it was necessary to calculate values from data for these ions at infinite dilution and 25°C. (14, 15, 27) on the following assumptions.

1. That sodium salicylate is a strong electrolyte and completely dissociated.
2. That the transference numbers of the Na^+ and salicylate ion are practically constant with variation in temperature.
3. That the transference numbers of Na^+ and salicylate ion are affected only slightly by change in concentration.

Assumption 1 is probably true since sodium salicylate has most of the properties of a strong electrolyte. However, it is well known that assumptions 2 and 3 are not valid in general (35). Independent evidence for the validity of 3 in the case of sodium salicylate was obtained from the stationary character of the buffer-concentration boundaries in routine electrophoresis experiments. These boundaries remain practically motionless over a period of four hours and this can only occur when the transference numbers of the ions are the same in both solutions (57). The correctness of assumption 2 is more questionable but Getman and Daniels (14) and Glasstone (15) show that the variation is small, except for the most highly hydrated ions, over a wide temperature range. Therefore, for a first approximation and as a working hypothesis, it is believed that these two simplifications are justified. Using these assumptions and the published values for the absolute mobility of the sodium ion at 25° and

infinite dilution (14) the relative mobilities of the three ions involved were calculated.

The determination of the change in refractive index at the buffer-concentration boundary per unit concentration of protein proved more difficult. The lack of a hollow prism made it impossible to apply Longsworth's procedure (30, 31). Moreover, because of the extremely small quantities involved, efforts to measure them directly failed. It was necessary to resort to an indirect method as follows: Under the same conditions of temperature, magnification, type of cell as in electrophoresis, large schlieren patterns due to a boundary between water and a .1 molar solution of sucrose in water were obtained. The method was essentially that used by Rothen (45) in his adaptation of the electrophoretic apparatus for studying diffusion of ribonuclease. Since the areas of these patterns as measured by a planimeter are proportional to the differences in the refractive index of the two solutions at the boundary, which can be measured with a refractometer, it is possible to associate a unit area under a curve with a fixed difference in refractive index at the boundary (all other conditions remaining constant). Therefore, the area under a buffer concentration boundary peak, as obtained in routine measurements, multiplied by the constant obtained as above gives the value of the refractive index change at the boundary. This change in refractive index can then be correlated with the protein concentration of the solution in the cell as determined by a

infinite dilution (λ_0) the relative mobilities of the three ions involved were calculated.

The determination of the change in refractive index at the buffer-concentration boundary per unit concentration of protein proved more difficult. The lack of a Tiselius peak made it impossible to apply Longsworth's procedure (30, 34). Moreover, because of the extremely small gradients involved, efforts to measure them directly failed. It was necessary to resort to an indirect method as follows: Under the same conditions of temperature, magnification, type of cell as in electrophoresis, large diffusion patterns due to a boundary between water and a .1 molar solution of sucrose in water were obtained. The method was essentially that used by Tiselius (45) in his elucidation of the electrophoretic apparatus for studying diffusion of ribonuclease. Since the areas of these patterns as measured by a planimeter are proportional to the differences in the refractive index of the two solutions at the boundary, which can be measured with a refractometer, it is possible to associate a unit area under a curve with a fixed difference in refractive index at the boundary (all other conditions remaining constant). Therefore, the area under a buffer concentration boundary peak, as obtained in pooling measurements, multiplied by the constant obtained as above gives the value of the refractive index change at the boundary. This change in refractive index can then be correlated with the protein concentration of the solution in the cell as determined by a

Kjeldahl procedure. Since the relationship is linear for dilute solutions of proteins (31), the slope of this line gives the refractive index change per unit concentration of protein at the buffer-concentration boundary, which is the parameter required.

After this investigation was nearly complete, it became apparent that it would be useful to know the density of dry gluten. No estimate of this constant could be found so an attempt was made to determine it by the methods of Neurath and Bull (39) for egg albumin. Their materials and technique were followed closely throughout except in the preparation of the sample.

As a means of estimating the relative importance of starch and electrolyte contamination in the final product, the gluten for the density determination was prepared by two methods, each represented by five independent samples.

In method 1, the gluten ball as obtained from 20 g. of ether-extracted flour was merely washed extremely thoroughly with distilled water until only the faintest traces of starch were apparent in a sedimentation test. In general, this required more than an hour per sample. The thoroughly washed gluten was then frozen and ground, while frozen, to a coarse powder. The water was removed from this frozen gluten by lyophilization in a high vacuum over P_2O_5. Throughout this process, the sample was kept frozen by solid CO_2. The resulting

coarse granules which retained the natural color of gluten were powdered in a mortar and stored over P_2O_5 until used. All the original properties of the gluten could be recovered by the addition of water to this powder.

In method 2, the gluten was washed from 20 g. of ether-extracted flour in the ordinary manner and dispersed for three days in sodium salicylate. The undispersed material was then centrifuged off and the opaque solution, now considered to be free from starch, made up to 20% saturation with magnesium sulphate. The gluten precipitated by the magnesium sulphate was collected on a stirring rod and washed as thoroughly as possible with distilled water to remove the electrolytes. It was then frozen and lyophilized in the same manner as for the undispersed series.

Throughout this study, all protein concentrations were estimated by a micro-Kjeldahl apparatus accurate to $\pm 1\%$ of the total nitrogen present.

RESULTS

Preliminary Experiments

Orientation Experiment on Egg Albumin

Before undertaking work on gluten, it was thought advisable to check the apparatus and technique by duplicating previous work on a well known protein. For this experiment, the work of Tiselius on egg-albumin (58) was taken as reference except that the egg albumin was prepared according to the method of Kekwick and Cannan (20). The results are plotted in Fig. 1.

It is evident from the graph that the agreement between the observed pH-mobility curve and that of Tiselius is not as good as might be hoped for. The isoelectric point is shifted 0.15 pH units to the left as compared with the Tiselius value, which is three times greater than the error involved in reading the pH meter. Moreover, tests showed this discrepancy to be reproducible. In spite of this, these results were taken as indicating the general reliability of the technique, apparatus and methods of calculation used because the slopes of the two curves are identical. This could be so only if there were no serious mistakes in methods of observation and calculation. It was assumed that the difference in isoelectric points was

Preliminary Experiments

Orientation Experiment on Rec Lobster

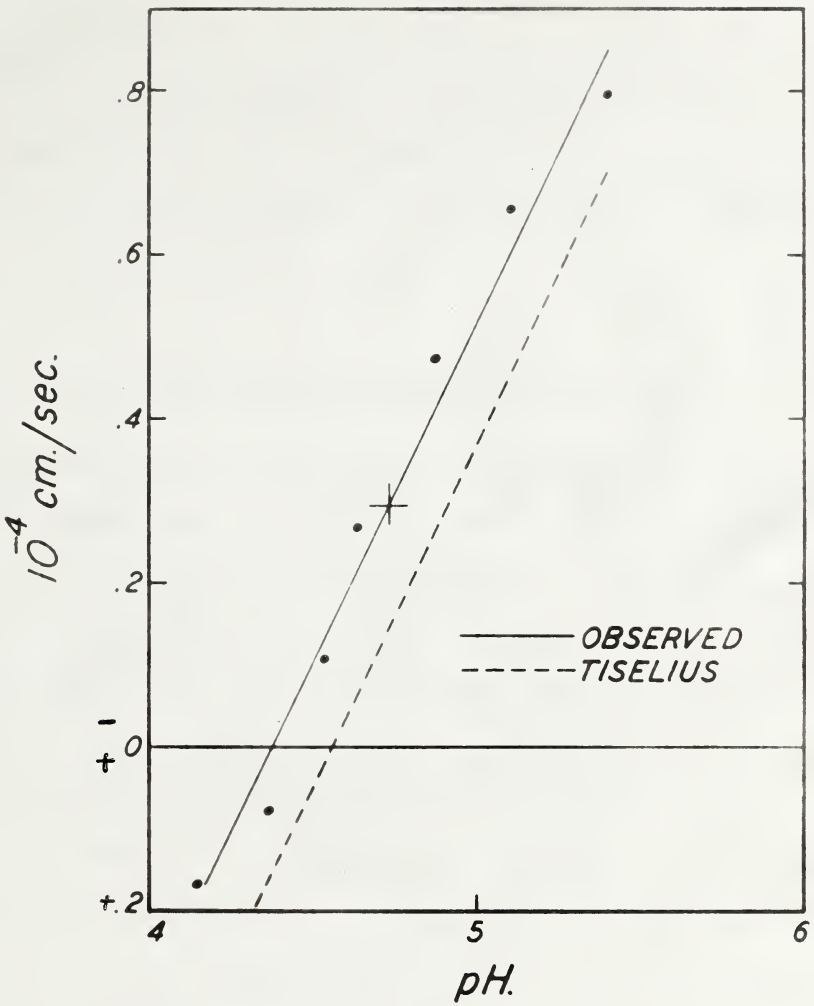


Figure 1

A comparison of the observed pH-mobility curve for egg-albumin in .02 M acetate buffer at 20°, with that of Tiselius for the same protein under the same conditions.

due to small differences in the original material or to differences in preparation. It is unlikely that the difference is due to errors in the standard buffer solution because this was made up carefully according to Clark (8) and checked against commercial buffer tablets.

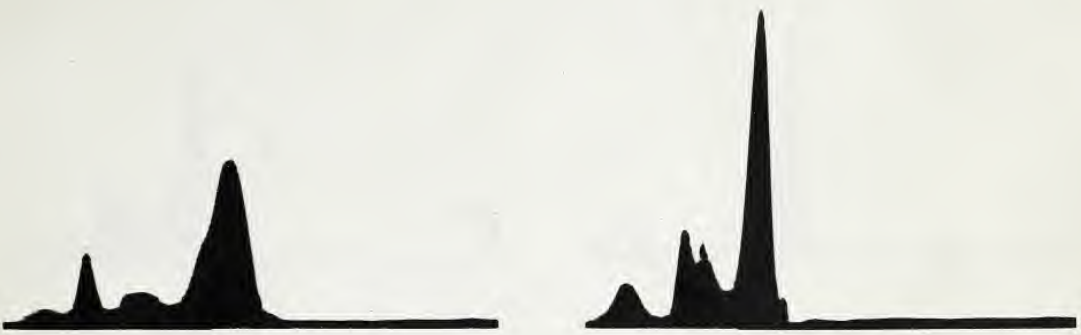
Effect of Sodium Salicylate on Egg White Proteins

Since sodium salicylate is a denaturing agent for some proteins (4) and must be used in rather high concentrations for gluten, the possibility of its altering or covering up some of the properties of the protein must be borne in mind. Rose and Cook (44) have shown that this reagent has only a slight effect on the properties of gluten as compared with the more usual dispersing agents, and Spencer and McCalla have demonstrated that there is no permanent alteration in the character of gluten after dispersion in salicylate (55). However this does not prove that there is not a temporary modification of the surface properties, particularly surface charge densities, while the gluten is dispersed. To demonstrate the effect of sodium salicylate solutions on a well characterized protein (32) the patterns of egg white in this reagent and in Sørensen's phosphate buffer, which is widely used as a buffering agent, were obtained. The patterns for this mixture of proteins are given at .1 M and at .5 M for both solutions in Fig. 2 and Fig. 3 respectively. An inspection of these patterns

due to small differences in the original material or to differences in preparation. It is unlikely that the difference is due to errors in the measured buffer solution because this was made up carefully according to Clark (5) and checked against commercial buffer tablets.

Effect of Protein Solutions on Tube Breaking

Under soaking solution is a breaking agent for some proteins (4) and must be used to reduce high concentrations for gluten. The possibility of its altering or covering up some of the properties of the protein must be borne in mind. Jago and Cook (10) have shown that this reagent has only a slight effect on the properties of gluten as measured with the mercurial dispersion agents, and Spencer and Mottram (11) have demonstrated that there is no permanent alteration in the characteristics of gluten after dispersion in sodium (10). However this does not prove that there is not a temporary modification of the surface properties, particularly surface coating materials, while the gluten is dispersed. To determine the effect of sodium sulphate solutions on a well characterized protein (4) the particle of egg white in this reagent was in Wintner's phosphate buffer, which is already used as a soaking agent, were obtained. The particles are only stainable of proteins are given at .1 % and at .5 % for basic solutions in fig. 1 and fig. 2 respectively. An inspection of these pictures



(a) (b)

Figure 2

Electrophoresis patterns of egg-white in
(a) .1 M phosphate buffer,
(b) .1 M sodium salicylate,
both at pH 6.9. Protein conc. 1.0%.
Time, 60 minutes.

(a) (b)

Figure 3

Electrophoresis patterns of egg-white in
(a) .5 M phosphate buffer,
(b) .5 M sodium salicylate,
both at pH 7.0. Protein conc. 1.0%.
Time, (a) 200 minutes,
(b) 330 minutes.

shows that the number of components of egg-white in sodium salicylate is the same as that in phosphate buffer. Moreover, the separation of components in sodium salicylate is equally as sharp as that in phosphate. A comparison of patterns at the two concentrations of both reagents indicates that the only effect of the increased ionic strength is a reduction in mobility as might be expected. From this, it was concluded that for the proteins of egg-white sodium salicylate is at least as satisfactory a buffer as Sørensen's phosphate. Unfortunately a similar direct comparison of the two solutions for gluten is not possible owing to the insolubility of gluten in most neutral solutions. The assumption has to be made that sodium salicylate has no more specific effects on gluten than on egg-white proteins.

The Effect of Acetic Acid, Sodium Hydroxide and Urea Solutions as Dispersing Agents.

The previous experiment suggested that it would be useful to know the behaviour of gluten in the other common dispersing agents, acetic acid, dilute sodium hydroxide and 30% urea. Representative patterns for gluten in .1 N acetic acid, .1 N NaOH and 30% urea are given in Fig. 4. For obvious reasons, no common conditions of pH, field strength or ionic strength are possible.

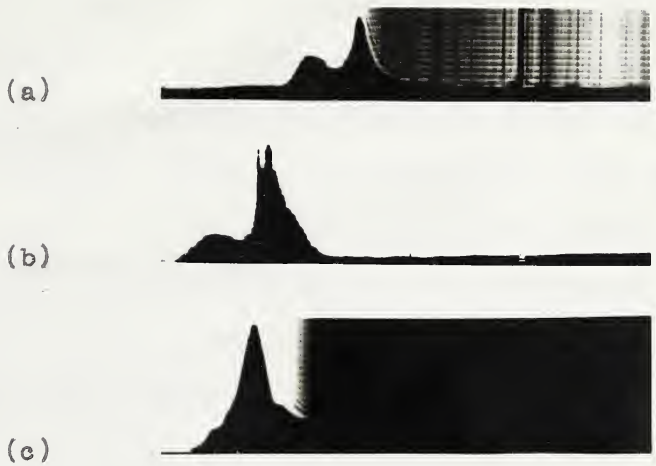


Figure 4

Electrophoresis patterns for gluten in
(a) .1 N acetic acid,
(b) .1 N sodium hydroxide,
(c) 30% urea.

As indicated by these patterns, gluten in acetic acid is markedly heterogeneous; at least two components are distinguishable and there are some indications of others. This is in agreement with the findings of Schwert, Putnam and Briggs (49) for gliadin. The heterogeneity is apparent in the NaOH solution but to a much smaller degree. In urea, however, gluten shows only one component which moves very slowly, probably as a result of the very high viscosity of the medium. The poor pattern obtained for urea was caused by the great opalescence and consequent low light transmission of the solutions.

The very different electrophoretic behaviour of gluten in these reagents can be correlated with the known specific effects of the reagents on other properties of gluten. Cook and Rose (11) found that acetic acid and sodium hydroxide caused marked hydrolysis of the gluten which was reflected in the viscosity of its solutions, while urea had practically no hydrolytic effect and may have had a coagulating tendency. Thus the heterogeneity of gluten in acetic acid and in sodium hydroxide is probably due to the presence of artifacts. However, it must be noted that Cook found the effect of sodium hydroxide on gluten to be much greater than that of acetic acid which is the reverse of the situation indicated by our patterns.

Main Electrophoresis Experiments

Following the completion of the preliminary experiments and a number of trial runs, pH-mobility curves for both the whole gluten and the molecularly dispersed gluten were determined. It was, of course, possible to obtain electrophoretic patterns for each series at the same time. The results of this study are given in Tables I and II and are plotted in Fig. 5 and Fig. 6.

Table I

Mobility of Whole Gluten as a Function of pH

pH	Mobility, cm/sec/volt X 10^4	pH	Mobility cm/sec/volt X 10^4
4.07	.348	6.06	.305
4.24	.287	6.23	.377
4.52	.344	6.28	.329
4.81	.330	6.45	.333
4.90	.279	6.52	.356
5.23	,300	6.53	.365
5.30	.285	6.54	.390
5.56	.299	6.60	.356
5.58	.347	6.70	.367
5.66	.341	6.79	.345
5.70	.362	6.86	.326
5.80	.395	7.09	.279
5.81	.358	7.19	.349
5.88	.309	8.19	.420
6.05	.376	8.25	.380

Main Thermodynamic Investigations

Following the investigation of the preliminary experiments and a number of small ones, thermodynamic curves for both the whole piston and the motionless cylinders were determined. It was, of course, possible to obtain electromotive pulsations for each series on the same disc. The results of this study are given in Tables I and II and are plotted in Fig. 3 and 4.

Table I

Mobility of Whole Piston as a Function of ω

ω	Mobility $\frac{cm/sec}{volt \times 10^{-6}}$	ω	Mobility $\frac{cm/sec}{volt \times 10^{-6}}$
.07	.340	6.06	.365
1.25	.329	6.25	.371
1.41	.341	6.41	.376
1.62	.345	6.62	.379
1.80	.379	6.80	.380
2.00	.390	7.00	.385
2.20	.391	7.20	.390
2.56	.399	7.56	.395
2.80	.400	7.80	.396
3.00	.402	8.00	.398
3.20	.405	8.20	.400
3.40	.406	8.40	.402
3.60	.408	8.60	.404
3.80	.410	8.80	.406
4.00	.412	9.00	.408
4.20	.414	9.20	.410
4.40	.416	9.40	.412
4.60	.418	9.60	.414
4.80	.420	9.80	.416
5.00	.422	10.00	.418
5.20	.424	10.20	.420
5.40	.426	10.40	.422
5.60	.428	10.60	.424
5.80	.430	10.80	.426
6.00	.432	11.00	.428

TABLE II

Mobility of Molecularly Dispersed Gluten
as a Function of pH

pH	Mobility, cm/sec/volt X 10^4	pH	Mobility, cm/sec/volt X 10^4
4.66	.312	6.43	.306
4.85	.318	6.52	.320
5.02	.271	6.64	.367
5.27	.309	6.83	.328
5.54	.265	6.90	.320
5.60	.274	7.09	.344
5.90	.248	7.60	.406
6.08	.297		

Representative electrophoresis patterns for whole gluten and molecularly dispersed gluten are given in Fig. 7 and Fig. 8 respectively.

Table II

Mobility of Molecularly Dispersed Dyes
as a Function of pH

pH	Mobility x 10^4	pH	Mobility x 10^4
4.66	.312	6.17	.000
4.85	.318	6.62	.250
5.05	.321	6.85	.000
5.27	.320	6.93	.250
5.45	.305	6.98	.260
5.60	.304	7.09	.260
5.90	.302	7.60	.000
6.09	.297		

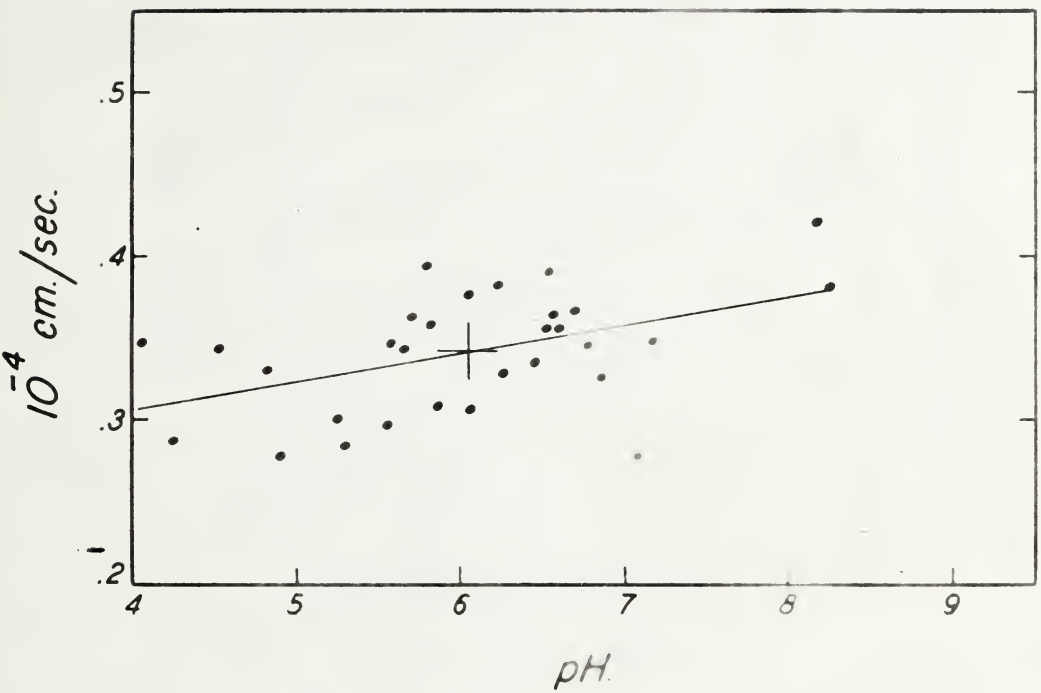


Figure 5

Mobility of whole gluten as a function of pH in 0.5 μ sodium salicylate.

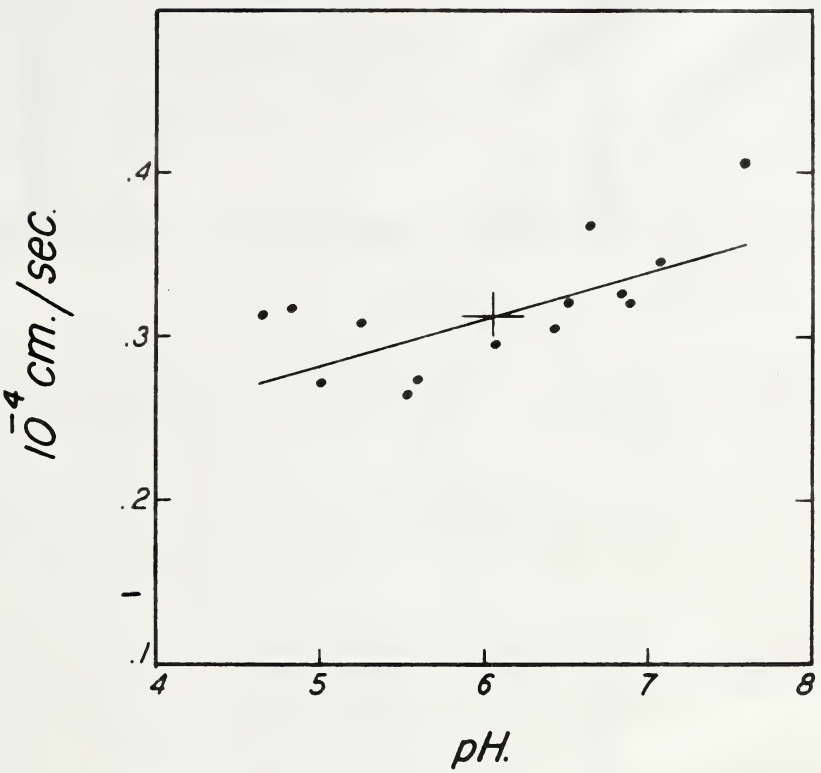


Figure 6

Mobility of molecularly dispersed gluten as a function of pH in 0.5 μ sodium salicylate.

Figure 7

Electrophoresis patterns of whole gluten in 0.5 μ sodium salicylate. Field strength 2.3 volts/cm. Time 250 minutes. Protein conc. .65%; pH 6.3 and 7.2

Figure 8

Electrophoresis patterns of molecularly dispersed gluten in 0.5 μ sodium salicylate. Field strength 2.3 volts/cm. pH 5.0. Time 250 minutes. Protein conc. 0.5%.

A study of Figures 7 and 8 shows that both the whole gluten and the molecularly dispersed portion are electrically homogeneous in sodium salicylate. The patterns are asymmetrical to a small degree in some cases but no patterns were obtained in which a separation of the peak could not be correlated with an error in technique. The only conclusion possible is that in sodium salicylate all fractions of gluten are essentially the same electrostatically. This does not apply to all portions of the nitrogenous constituents of wheat, however. It will be noticed that the patterns exhibit a very small peak migrating ahead of the main boundary. This was a constant feature of all patterns and its presence is proof that if major components are present in gluten, this technique should discover them. That the small peak or boundary is not a major fraction of gluten is proved by its size alone; it represents only a very small fraction of the total protein present and therefore cannot be important in the complete complex. It has been identified tentatively as an impurity in the sample due to the presence of non-gluten protein (37) [See Appendix II] .

Figures 5 and 6 show that within the pH range studied, 4.00 to 8.25, gluten carries a negative charge. Moreover, the size of this charge is remarkably insensitive to changes in pH. This is shown by the very low slope of the regression line. However, this low slope is probably due to the fact that it was not possible to work in the isoelectric range because

salicylic acid precipitates from the solution at pH values less than 4.0. All pH-mobility curves approach zero slope at hydrogen-ion concentrations remote from the isoelectric point. Independent evidence for the existence of an isoelectric point for gluten in sodium salicylate at a pH less than 4.0 was given by the strong tendency for the gluten to precipitate at pH values less than 5.0. Below pH 5.0 it was difficult to keep the gluten in solution although this tendency to settle out was not accompanied by the precipitation of salicylic acid until pH 4.0 was reached. This very low isoelectric point of gluten in sodium salicylate will be referred to again in connection with the high value of the surface charge density.

A comparison of Fig. 5 with Fig. 6 will show that the slopes of the regression lines are approximately the same for whole gluten and for the molecularly dispersed fraction. This is confirmed by statistical analysis* and this fact corroborates the suggestion from the electrophoresis patterns that the surface charge characteristics are the same in both cases, since they react similarly to changes in the medium. Statistical analysis also proves** that the mean mobility at any given pH is lower for the molecularly dispersed fraction than for whole gluten. Since the mean particle size is lower for the molecularly dispersed fraction (37) and the evidence given above indicates that the surface charge densities are approximately equal, this lower mobility must be a reflection of a larger frictional

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*t = 0.98 **t = 2.36

ratio $\frac{f}{f_o}$. This is in agreement with the observation of McCalla and Gralén that the most soluble fraction of gluten has the largest frictional coefficient. Assuming constant hydration, this in turn must mean that the particles making up the most soluble portion of gluten have the greatest length to breadth ratio.

These two experiments compel two conclusions; 1. That in sodium salicylate, gluten is an electrostatically homogeneous substance, 2. That the isoelectric point of gluten in sodium salicylate is somewhere below pH 4.0. These conclusions concerning the nature of gluten are so much at variance with the results of Schwert, Putnam and Briggs (49) on gliadin that it was worthwhile to repeat their study using gluten from our flour. It proved possible to duplicate their work in all respects but one. Even at field strengths in excess of those mentioned in their report no multiple separation of the two main components into lesser complexes could be obtained. All other observations made by these authors were confirmed, however, proving that the source of the discrepancy does not lie in the raw material used. We are forced to the conclusion that the fundamental mechanisms of dispersion in acetic acid and in sodium salicylate must be different.

While this study was in progress, Scallet (47) published a report on the electrophoretic analysis of zein, in which he observed that as the ionic strength increased, separation of components in the mixture decreased. It has been

ratio $\frac{\lambda}{D}$. This is in agreement with the observation of

suggested that this suppression is the cause of the apparent electrophoretic homogeneity of gluten. To test this point as far as physical conditions would permit, a dispersion of gluten in 0.5 μ salicylate was diluted until the protein was on the verge of precipitation. The final concentration of sodium salicylate was 0.3 M. An electrophoretic analysis was then made on the sample at this concentration. The results are shown in Fig. 9.

It will be observed that these two plates give no more evidence for the heterogeneity of gluten than do those conducted at 0.5 μ. One main peak only is present in each pattern. Since 0.3 M is still a fairly concentrated solution, this experiment is not conclusive, but taken in conjunction with the presence of the non-gluten protein boundaries, it is a strong indication that the homogeneity of gluten is not due to suppression by high salt concentration.

Figure 9

Electrophoresis patterns of whole gluten in 0.3 M sodium salicylate. Field strength 3.8 volts/cm. pH 6.0. Time 250 minutes. Protein conc. 0.35%.

It was also thought that the failure to resolve components in gluten might be simply due to the low mobility in the high salt concentrations. If this were so, increasing the current-time product should tend to separate components. Accordingly, an experiment was conducted on whole gluten in 0.5 μ sodium salicylate using as high a current as the thermostat would allow for as long a period as the electrode capacity would permit. The results are shown in Fig. 10.

These patterns are in every way comparable to those obtained under the less rigourous conditions and give no support to the above suggestion.

Summing up all the evidence, the most reasonable interpretation is that sodium salicylate does not seriously affect the separation of components. The work with egg-white proteins, non-gluten protein constituents and with low concentrations of electrolytes supports this view. It must be pointed out, nonethe-less, that this evidence is suggestive only, not conclusive. Sookne and Harris (53) working with the proteins of silk have shown that the more complex ions such as phthalate and picrate are preferentially adsorbed, sometimes to the point where the isoelectric point disappears from the range studied. If gluten constituents should selectively adsorb salicylate ions, it might raise their electronegativity to a point where smaller initial differences are not distinguishable. This question deserves further study.

Figure 10

Electrophoresis patterns of whole gluten in 0.5 μ sodium salicylate. Field strength 1.7 volts/cm. pH 6.7. Protein conc. .5%. Time 600 minutes.

Diffusion

TABLE III

Diffusion Constant of Molecularly Dispersed Gluten at 2.0°C.

Run No.	Time (min.)	D X 10^7
1	510	2.13
	1056	2.06
2	564	1.98
	762	1.89
	1333	1.97
3	518	2.13
	787	1.96
4	267	2.00
	503	1.97
5	258	2.28
	698	1.97
	1371	1.91
6	328	1.98

In Table III are given the values obtained from thirteen determinations of the diffusion constant of molecularly dispersed gluten at 2.0° ± .05 with the corresponding period of diffusion. It will be observed that, in general, the longer periods are associated with the lower diffusion constants. This is because some slight mixing of solutions is unavoidable when forming the boundaries. The effect of mixing is to raise the apparent diffusion constant but it is obvious that the effect will approach zero as the time of diffusion approaches infinity. In order to approximate this condition the regression line of

Diffusion

Table III

Estimated Constants of Equation(1)
Diffusion Times at $T_0 = T_0$.

Run No.	Time (min.)	$D \times 10^5$
1	720	2.12
	1050	2.06
2	540	2.26
	740	2.28
	1475	2.27
3	415	2.32
	707	2.25
4	340	2.20
	504	1.97
5	295	2.20
	606	1.97
	1372	1.91
6	325	1.98

In Table III are given the values obtained from numerous determinations of the diffusion constant of sodiumethyl silicoformate at $T_0 = 25.0^\circ$ C. by which the corresponding period of diffusion. It will be observed that, in general, the longer periods are associated with the lower diffusion constants. This however were slight plotting of solutions in unsymmetrical containing the constituents. The effect of mixing is to give the apparent diffusion constant but it is obvious that the effect will approach zero as the time of diffusion approaches infinity. It seems to be sufficient only condition the determination of

a plot of $\frac{1}{t}$ against the diffusion constant was calculated and the "true" diffusion constant taken as the value at which $\frac{1}{t} = 0$. Corrected in this manner, the mean diffusion constant of molecularly dispersed gluten in 0.5 μ sodium salicylate at 2.0° was estimated as $(1.91 \pm .09) \times 10^{-7}$ moles/cm^2/sec.

It cannot be reiterated too frequently that the value of the diffusion constant obtained here is a mean value for the molecularly dispersed portion of gluten and therefore any particular part of this fraction may be expected to yield values which differ from the figure given. Since gluten is a polydisperse system (37) the same limitation applies to practically all numbers which are used to describe any appreciable part of the complex. These numbers are intended to give a reliable general description of the protein; not to furnish exact magnitudes for a particular case.

Density of Gluten

TABLE IV

Density of Dry Gluten at 20°C, g./c.c.

Series	Sample	Density	Mean
Washed	1	1.299*	
	2	1.312	
	3	1.311	1.310
	4	1.310	
	5	1.309	
Reprecipitated	1	1.296	
	2	1.292	
	3	1.288	1.291
	4	1.291	
	5	1.288	

*Rejected on a statistical basis according to Willard and Furman, Elementary Quantitative Analysis, p.67.

In Table IV are given the results of a determination of the density of dry gluten, prepared by the two methods described previously. It is evident from inspection that the two series must differ and this opinion is sustained by statistical analysis*. Since the washed samples were known to contain very small amounts of starch which has a density of 1.50 (16), the mean of the values for the reprecipitated series was taken as the better estimate of the density of dry gluten.

* t = 10.9

Density of Dry Gluten

TABLE IV

Density of Dry Gluten at 30°C. [illegible]

Series	Sample	Density	Mean
Wetted	1	1.[illegible]	
	2	1.[illegible]	
	3	1.[illegible]	1.[illegible]
	4	1.[illegible]	
	5	1.[illegible]	
Superdried	1	1.[illegible]	
	2	1.[illegible]	
	3	1.[illegible]	1.[illegible]
	4	1.[illegible]	
	5	1.[illegible]	

[illegible]
to Milled and Cereal Chemistry Chemical
Analysis, p. 1[illegible].

In Table IV are given the results of a determination of the density of dry gluten, prepared by the two methods described previously. It is evident from inspection that the two series must differ and this opinion is confirmed by statistical analysis. Since the wetted samples were known to contain very small amounts of extract which was a density of 1.[illegible], the mean of the values for the superdried series is taken as the better estimate of the density of dry gluten.

[illegible]

* t = 10.[illegible]

Data for Electrical Charge on Gluten

TABLE V

Change in Refractive Index at Buffer-Concentration Boundary as a Function of Nitrogen Content of the Solution

Mgm. N/c.c.	Refractive Index Change X 10^6
.10	25
.12	58
.28	104
.33	110
.44	185
.50	164
.58	189
.63	246
.70	252
.77	264
.88	287
.88	296

In Table V are given the changes in refractive index at the buffer-concentration boundary corresponding to various nitrogen levels of the solution. These values are plotted in Fig. 11.

The calculated regression equation is $\Delta n = 10 \times 10^{-6} + 331.5 \times 10^{-6}$ mgm. N/c.c. with a standard error of estimate of 17×10^{-6}. Thus, within the limits of experimental error, the line passes through the origin as it should. The slope of this line is $(331.5 \pm 18.5) \times 10^{-6}$ per mgm. N. per c.c., which, assuming a nitrogen factor for endosperm proteins of wheat of 5.70 (19) is equal to $(581.6 \pm 32.5) \times 10^{-6}$ per gram of protein per 100 ml.

Table for Binomial Coefficients

Number of Combinations of n Objects Taken r at a Time

n	r	C_r^n
1	0	1
	1	1
2	0	1
	1	2
	2	1
3	0	1
	1	3
	2	3
	3	1
4	0	1
	1	4
	2	6
	3	4
	4	1
5	0	1
	1	5
	2	10
	3	10
	4	5
	5	1
6	0	1
	1	6
	2	15
	3	20
	4	15
	5	6
	6	1
7	0	1
	1	7
	2	21
	3	35
	4	35
	5	21
	6	7
	7	1
8	0	1
	1	8
	2	28
	3	56
	4	70
	5	56
	6	28
	7	8
	8	1
9	0	1
	1	9
	2	36
	3	84
	4	126
	5	126
	6	84
	7	36
	8	9
	9	1
10	0	1
	1	10
	2	45
	3	120
	4	210
	5	252
	6	210
	7	120
	8	45
	9	10
	10	1
11	0	1
	1	11
	2	55
	3	165
	4	330
	5	462
	6	462
	7	330
	8	165
	9	55
	10	11
	11	1
12	0	1
	1	12
	2	66
	3	220
	4	495
	5	792
	6	924
	7	792
	8	495
	9	220
	10	66
	11	12
	12	1
13	0	1
	1	13
	2	78
	3	286
	4	715
	5	1287
	6	1716
	7	1716
	8	1287
	9	715
	10	286
	11	78
	12	13
	13	1
14	0	1
	1	14
	2	91
	3	364
	4	1001
	5	2002
	6	3003
	7	3432
	8	3003
	9	2002
	10	1001
	11	364
	12	91
	13	14
	14	1
15	0	1
	1	15
	2	105
	3	455
	4	1365
	5	3003
	6	5005
	7	6435
	8	6435
	9	5005
	10	3003
	11	1365
	12	455
	13	105
	14	15
	15	1
16	0	1
	1	16
	2	120
	3	560
	4	1820
	5	4368
	6	8008
	7	11440
	8	12870
	9	11440
	10	8008
	11	4368
	12	1820
	13	560
	14	120
	15	16
	16	1
17	0	1
	1	17
	2	136
	3	680
	4	2380
	5	6188
	6	12376
	7	19448
	8	24310
	9	24310
	10	19448
	11	12376
	12	6188
	13	2380
	14	680
	15	136
	16	17
	17	1
18	0	1
	1	18
	2	153
	3	816
	4	3060
	5	8568
	6	18564
	7	31824
	8	43758
	9	48620
	10	43758
	11	31824
	12	18564
	13	8568
	14	3060
	15	816
	16	153
	17	18
	18	1
19	0	1
	1	19
	2	171
	3	969
	4	3876
	5	11628
	6	27132
	7	50388
	8	75582
	9	92378
	10	92378
	11	75582
	12	50388
	13	27132
	14	11628
	15	3876
	16	969
	17	171
	18	19
	19	1
20	0	1
	1	20
	2	190
	3	1140
	4	4845
	5	15504
	6	38760
	7	77520
	8	125970
	9	167960
	10	184756
	11	167960
	12	125970
	13	77520
	14	38760
	15	15504
	16	4845
	17	1140
	18	190
	19	20
	20	1

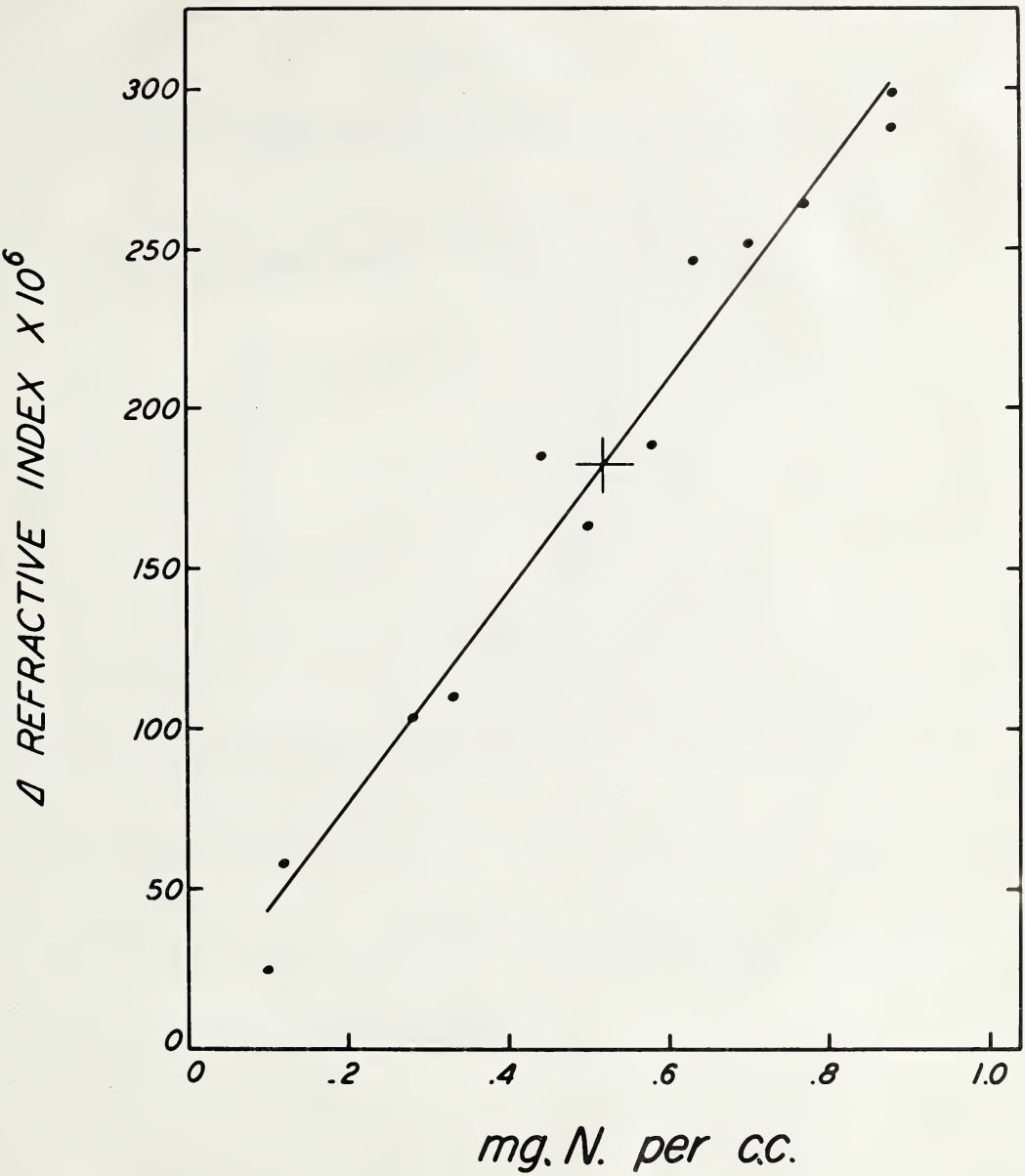

Figure 11

Change in refractive index
at the buffer concentration boundary
as a function of nitrogen content
of the solution.

TABLE VI

Refractive Index of Sodium Salicylate Solutions as a Function of Equivalent Concentration

Equivalents/liter	Refractive Index
0	1.3339
.05	1.3356
.10	1.3373
.15	1.3390
.20	1.3408
.25	1.3424
.30	1.3441
.35	1.3459
.40	1.3476
.45	1.3492
.50	1.3510

Table VI lists the refractive indices of progressively varying concentrations of sodium salicylate in water. These data are plotted in Fig. 12. The slope of the regression line in these units is equal to the K_{AR} constant in the formula of Longsworth. Since the regression equation is n = 1.3339 + .03417 equivalents the slope is equal to .03417 $\pm$ .00012 per equivalent of sodium salicylate.

TABLE VI

Corrective Layer of Sodium Polyplate Solutions
as a Function of Equivalent Concentration.

Equivalents/Liter	Corrective Layer
0	1.4329
.04	1.3554
.10	1.3077
.16	1.2[illegible]
.20	1.2[illegible]
.24	1.2[illegible]
.30	1.2[illegible]
.36	1.2[illegible]
.40	1.2[illegible]
.44	1.2[illegible]
.48	1.2[illegible]
.52	1.2[illegible]
.60	1.2[illegible]

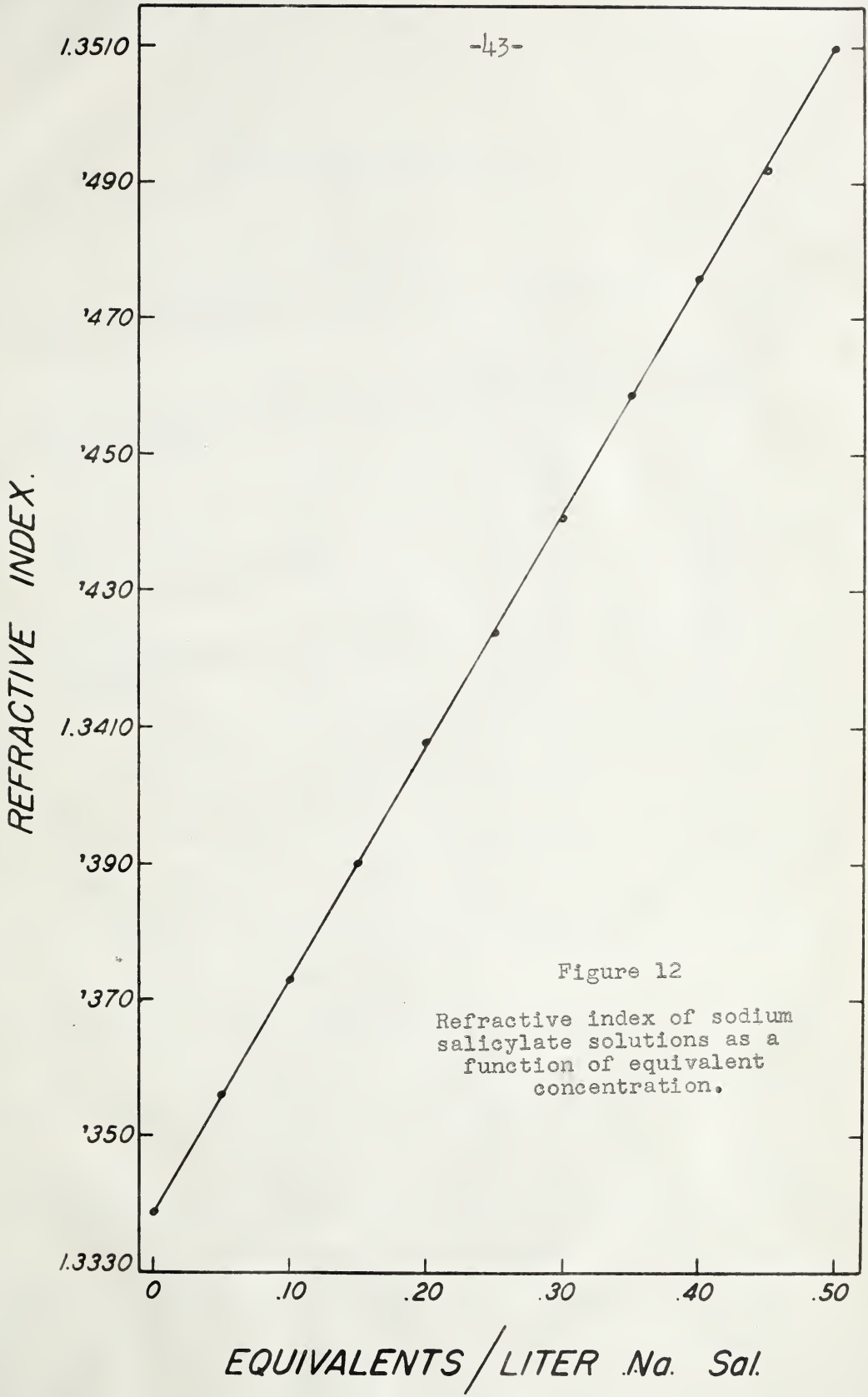

Figure 12

Refractive index of sodium salicylate solutions as a function of equivalent concentration.

Viscosities of Solutions

TABLE VII

Viscosities of Solutions, 2.0°C.

Series	Sample	Viscosity (centipoises)	Prot. Conc.
0.5 μ Sodium Salicylate	1	2.056	--
	2	2.054	--
Whole Gluten	3	2.503	.86%
	4	2.482	.87%
	5	2.546	.98%
	6	2.461	.84%
Molecularly Dispersed Gluten	7	2.310	.61%
	8	2.312	.62%
	9	2.319	.62%
	10	2.320	.61%

In Table VII are given the viscosities in centipoises at 2.0° ± .05 of the various solutions of gluten in sodium salicylate used, and two values for the viscosity of 0.5 μ sodium salicylate as determined from independent solutions. Each value listed is the mean of at least five trials. The corresponding protein concentrations are given as calculated from mgm. of nitrogen per c.c. and represent the limiting factor in the accuracy of these determinations.

There is, as pointed out by Abramson, Moyer and Gorin (1, p.137), a difficulty in the physical interpretation of the viscosity as determined in the laboratory. In very dilute solutions of spherical proteins at their isoelectric point, the viscosity term occurring in the electrophoretic and

Viscosities of Solutions

Table VII

Viscosities of Solutions, $\eta \times 10^{2}$.

Series	Density	Viscosity (centipoises)	Temp.
0.5 M Sodium Methylate	1	1.0[illegible]	--
	2	1.0[illegible]	--
Methyl Ester	1	[illegible]	[illegible]
	2	[illegible]	[illegible]
	3	[illegible]	[illegible]
	4	[illegible]	[illegible]
	5	[illegible]	[illegible]
Methanol	1	[illegible]	[illegible]
	2	[illegible]	[illegible]
Diphenyl	4	[illegible]	[illegible]
Nitrobenzene	10	[illegible]	[illegible]

diffusion equations "is clearly that of the medium and is equivalent to the coefficient of viscosity as determined in the laboratory." When the solutions become more concentrated and particularly when the particles are both highly charged and highly anisometric, this is no longer true. As a result of the higher concentration, the Helmholtz double layers of the particles overlap and there is a mechanical interference of the particles with each other. This effect of the motion of all neighbouring particles upon the migrating particle is called the hydrodynamic effect by Abramson, Moyer and Gorin.

In addition, they distinguish two non-hydrodynamic interactions between protein molecules. One type occurs in highly concentrated protein solutions where the molecules lie so close together that their double layers become superimposed. This particular effect is not important for the interpretation of the equations to be given. Their second non-hydrodynamic effect, however, is quite important for the particular case of gluten and is a result of the definite orientation of highly anisometric, charged particles in a medium as a result of the electrostatic forces between them. These forces tend to arrange the molecules with their axes in a preferred direction. This means that additional work must be done to destroy this orientation on movement of the medium. As the above authors point out, the theory of these effects is largely undeveloped and those parts of it which have been considered are untested experimentally. For this reason, the adoption of any value for the viscosity

term in these equations has an element of arbitrariness. None-the-less, since these flow-resisting forces undoubtedly exist in the solutions and must be taken into account at least partially, the viscosity of the whole solution rather than that of the medium has been adopted as the closer approximation to the truth.

Calculated Results

Molecular Charge

The foregoing experimental data permit the calculation of the mean charge of the molecularly dispersed gluten particles as outlined previously. The constants used and the results of the application of Longsworth's formula are summarized in Table VIII.

TABLE VIII

Data for Calculation of Molecular Charge

$\Lambda_{(0.5\ \mu)}$ of sodium salicylate at 25° = 49.8

Λ_o of salicylate ion at 25° = 37.1

Λ_o of sodium ion at 25° = 50.1

Absolute mobility of sodium ion at infinite dilution and 25° = 5.2×10^{-4} cm/sec/volt.

Ratio of conductivity of 0.5 μ sodium salicylate at 2° and 25° = .557.

$n^{\beta}-n^{\gamma} = 331.5 \times 10^{-6}$ ρ = .570 g/100 ml.

$K_{AR} = 341.7 \times 10^{-4}$ r_A = +1.000

r_R = — .741 r_B = — .188

Mean charge per molecule = 34 on the assumption that mean molecular weight is 44,000 (37).

Differential Heating

Laboratory Theory

The foregoing experimental data permit the calculation of the mean change of the molecularly dispersed fluxes mentioned as outlined previously. The fundamental laws and the results of the application of Langmuir's Theorie are summarized in Table VIII.

Table VIII

Data for Calculation of Chemical Change

$\int_{0.5}^{1}$ of sodium molybdate at 25° = $\int_{0}^{1}$.

$\int_{0}^{1}$ of molybdate ion at 25° = $\int_{0}^{1}$.

$\int_{0}^{1}$ of sodium ion at 25° = $\int_{0}^{1}$.

Absolute mobility of sodium ion or Ionic diffusion rate U_{Na}^{o}
= 5.2×10^{-4} cm./sec./volt.

Ratio of conductivity of 0.5 M sodium molybdate to K^{+} and $SO_4^{=}$
= .557.

K_{Na} = $[0.5 \times 10^{-4}$ / = .520 $\times 10^{-4}$.

K_{MoO_4} = $[0.7 \times 10^{-4}$ / = .040

= — .017 = — .160

This calculation gives the mean "valence" of molecularly dispersed gluten at pH 6.00 in 0.5 μ sodium salicylate as 34. From the low slope of the curve shown in Fig. 6 it is evident that this is an estimate of molecular charge over a considerable range of pH. For a protein of molecular weight 44,000, this "valence" is high. Egg albumin with a comparable molecular weight varies from 0 to -14 over a similar interval. This is a further indication of possible specific ion effects of salicylate on gluten (53). The reversible adsorption of negative salicylate ions on the gluten micelle may raise the electronegativity of the molecule to the observed high value. This would also explain the displacement of the isoelectric point so far towards the acid range and the observed flattening of the pH-mobility curve.

Estimation of Molecular Weight From Diffusion and Viscosity Data

It is possible to check the internal consistency of the data listed by a calculation of the molecular weight of the protein. Polson (42) and Svedberg and Pedersen (56) have shown that if the diffusion constant, the partial specific volume and the frictional coefficient of a particle are known, a reasonably accurate estimate of the molecular weight is possible from the formula,

$$\frac{f}{f_o} = \frac{RT}{D} \cdot \frac{(4\pi N)^{\frac{1}{3}}}{6\pi\eta N(3\ M\ V)^{\frac{1}{3}}}$$

where $\frac{f}{f_o}$ = frictional coefficient

R = gas constant = 8.313×10^7

T = temperature ^oK

D = diffusion constant

η = viscosity of solution

N = Avogadro's number

M = molecular weight

V = partial specific volume.

Using the mean value of $\frac{f}{f_o}$ for the molecularly dispersed portion of gluten from McCalla and Gralén (37) and their value for V in 8% sodium salicylate (corrected for the difference in temperature according to Svedberg and Pedersen (56, Appendix II) with the constants listed in the foregoing tables, a molecular weight of 48,000 was found for molecularly dispersed gluten. This is in satisfactory agreement with the ultracentrifugal value of 44,000 (37) and indicates the general reliability of the constants used.

These data also furnish reliable evidence against the hypothesis that gluten is a highly hydrated sphere. It is possible to explain the extremely large observed $\frac{f}{f_o}$ value on the basis of a sphere with a thick shell of water. However, using the value of the diffusion constant given and the formula (1, p.130),

$$D = \frac{kT}{6\pi\eta r}$$

where D = diffusion constant

k = Boltzmann's constant

T = temperature ^oK

η = viscosity of solution

r = radius of particle

where $\frac{f}{f_o}$ = frictional coefficient

λ = gas constant = 8.314×10^7

T = temperature ^oK

κ = diffusion constant

ζ = viscosity of solution

β = Svedberg number

M = molecular weight

$\bar{v}$ = partial specific volume.

Using the mean value of $\frac{f}{f_o}$ for the molecularly dispersed protein of Lyman Duncan and Taylor (57) and Svedberg values for $\bar{v}$ in 50 sodium salicylate (corrected for the dilution by condensed water according to Svedberg and Pedersen (58, appendix A)) when one substitutes in the corresponding Svedberg, a molecular weight of 40,000 was found (as mentioned elsewhere). This is in satisfactory agreement with the ultracentrifuged value of 34,000 (57) and indicates the general reliability of the centrifuge used.

There are also further reliable evidence against the hypothesis that Bjerrum is a highly dispersed solvate. It is easily to calculate the extremely large observed $\frac{f}{f_o}$ value on the basis of a sphere with a thick sheet of water. However, using the value of the diffusion constant given and the formula (3, p.1201)

$$D = \frac{kT}{6\pi\zeta r}$$

where D = diffusion constant

k = Boltzmann's constant

T = temperature ^oK

ζ = viscosity of solution

r = radius of particle

r is found to be 45.6 Å. From the density data given, an unhydrated gluten molecule of molecular weight 44,000 corresponds to a sphere of radius 23.8 Å. Therefore in order to explain the observed diffusion constant by hydration of a sphere it is necessary to postulate the adsorption of a shell of water approximately 20 Å in thickness. Such a shell is extremely improbable on the basis of electrostatic forces alone (1, p.151) and therefore the data must be interpreted on the assumption of rather large asymmetry of the molecule. Such asymmetry may be represented by two models; (a) The oblate ellipsoid, (b) The prolate ellipsoid. Simha has shown that for proteins in general and for gliadin in particular, the prolate ellipsoid is the much more probable model (51). On the basis of this evidence, therefore, it is concluded that an elongated ellipsoid of revolution is a reasonable approximation to the shape of the gluten molecule.

Molecular Length-Breadth Ratio

The frictional coefficient $\frac{f}{f_o}$ has sometimes been used as a parameter for the estimation of the axial ratios of molecules. This procedure is not valid in general since the coefficient is a function of the degree of hydration of the molecule as well as its asymmetry. The two effects cannot be separated by a single measurement except for the special cases of zero hydration or completely spherical molecules (56). Polson (42) and more recently Simha (50) have given equations from which an

Molecular Length-Breadth Ratio

estimate of the axial ratios of anisometric molecules can be obtained from viscosity data. Polson's equation is an empirical generalization of Kuhn's equation (25) for rod shaped particles and he has shown (42) that it gives results in good agreement with experimental values. Simha's equation is an extension of Einstein's relation

$$\frac{u - u_o}{u_o} = \nu c$$

where u = viscosity of solution

u_o = " " solvent

c = volume fraction of solute

and ν is a shape factor,

to the case where the particles are prolate or oblate ellipsoids suspended in a medium. He too has demonstrated that results calculated on the basis of his equation are in good agreement with experiment (50).

Substituting the data from Table VII in Polson's equation,

$$\frac{u}{u_o} = 1 + 4.0cV + 0.098cVk^2$$

where c = conc. of protein in grams/c.c.

V = partial specific volume

k = axial ratio = $\frac{\text{Length}}{\text{Diameter}}$ of ellipsoid

gives a k value of 16.3 for molecularly dispersed gluten. Substitution of the same data in Simha's equation

$$\frac{u}{u_o} - 1 = cV\left[\frac{k^2}{15(\ln 2k - \frac{3}{2})} + \frac{k^2}{5(\ln 2k - \frac{1}{2})} + \frac{14}{15}\right]$$

leads to a k value of 17.1. Thus the results using the two methods are in essentially good agreement. Taking the mean of the two estimates gives a mean axial ratio of 16.7 for the molecularly dispersed gluten particle.

The good agreement of these two equations, while reassuring, must not be overstressed. It is to be expected that they would tend to the same value since both are based on similar assumptions; that is, on complete Brownian movement among rotational ellipsoids. As has been pointed out by Robinson (43), it is of paramount importance that the basis of these equations be realized. If complete Brownian movement does not exist and the particles are to some extent oriented, viscosity is reduced and therefore the apparent value of k is lowered. The tendency towards orientation is particularly strong in capillary viscosimeters with their high rates of shear. Moreover, orientation is much more probable with greatly anisometric particles. Therefore, the value 16.7 is to be regarded as a lower limit until it can be confirmed by improved methods.

In spite of these limitations, if Perrin's formula (41) for prolate ellipsoids is used to calculate the $\frac{f}{f_o}$ corresponding to a k value of 16.7, we obtain a frictional coefficient of 1.86 which is in satisfactory agreement with McCalla and Gralén's figure of 1.92 after the effects of possible hydration are considered.

$$\frac{\eta_0}{\eta} - 1 = \alpha\nu \left(\frac{1}{10\left(\frac{1}{\nu} - \frac{1}{3}\right)} + \frac{2}{3\left(15\alpha\nu - \frac{5}{2}\right)} + \frac{1}{15} \right)$$

Molecular Volumes and Radius

Abramson has shown (1, p.130) that the combination of diffusion data and frictional coefficient also permits an independent calculation of the molecular volume. Using the equation

$$\frac{f}{f_o} = \frac{kT}{D6\pi\eta V^{1/3}} \left(\frac{4\pi}{3}\right)^{1/3}$$

where k = Boltzmann's constant

η = viscosity of solution

V = molecular volume

D = diffusion constant

and the constants listed, we obtain a mean molecular volume for the gluten particle of 56 X $10^3 Å^3$. Calculation of the molecular volume of dry gluten from the density data listed also gives a value of 56 X $10^3 Å^3$. This agreement must be regarded as partly fortuitous, however, since the gluten molecule is almost certainly hydrated to some extent. The close approximation indicates strongly, however, that the hydration of gluten cannot be great or it would be reflected in a much larger molecular volume as calculated from diffusion data.

Combining the data for molecular volume from diffusion and the estimate of the axial ratio of the gluten particle from viscosity yields a radius of 9.3Å for the gluten molecule. For the reasons pointed out above, this figure is certainly a little low since the effects of hydration would tend to increase the radius. However, it is possible to get a second, independent estimate of the molecular radius from electrophoretic results.

Molecular Volume and Density

Combining the above (1, 2, 3, 4) gives the combination of diffusion data and intrinsic viscosities also provide an independent estimation of the molecular volume. Using the equation

$$\frac{f}{f_o} = \frac{kT}{6\pi\eta_0 r\sqrt{3}} \left(\frac{b}{3}\right) \qquad (3)$$

where b = molecular diameter

f = viscosity of solution

f_o = molecular volume

D = diffusion constant

and the Svedberg (1934) we obtain a mean molecular volume for the gluten particle of $2a \times 10^{-15}$. Calculation of the molecular volume of dry gluten from the density data listed also gives a value of $2a \times 10^{-15}$. This agreement must be regarded as merely fortuitous, however, since the given molecule is known certainly deformed to some extent. The close approximation indicates, however, that the diffusion of gluten cannot be greatly in excess of the molecular volume as calculated from diffusion data.

Combining the data for molecular volume from diffusion and the ellipsoid of the axial ratio of the gluten particle from viscosity yields a radius of 2.5×10^{-5} for the gluten molecule. For the reasons pointed out above, this figure is somewhat a little too large for molecular work, but can be interpreted for reason. However, it is possible to get a second, independent estimate of the molecular radius from electric conductivity measurements.

Abramson (1, p.128) gives the equation for electrophoretic mobility of a randomly oriented long rod as

$$v = \frac{\sigma}{\eta}\left(\frac{1}{\kappa} + r_i\right)\chi$$

where v = electrophoretic mobility

σ = charge density of particle

η = viscosity of solution

κ = Debye-Huckel parameter

r_i = average radius of ions in the ion atmosphere around the particle.

χ is a shape factor which for long rods has the form

$$\chi = \left[\frac{\frac{\kappa a}{(\kappa a+\kappa r_i)}\frac{K_o(\kappa a+\kappa r_i)}{K_1(\kappa a+\kappa r_i)} + \kappa a \ln\left(\frac{a+r_i}{a}\right)}{1+\kappa r_i}\right]\left[\frac{4}{F}\right]$$

where a = radius of rod

K_o and K_1 are Bessel functions

and $F = f(\kappa a, \kappa r_i)$

If the assumption is made that a prolate ellipsoid of great asymmetry approximates a long rod, this equation yields a molecular radius of 14Å. In contrast to the diffusion data, this value must certainly be too high since it corresponds to a molecular volume of several times that calculated from density data. Such a degree of hydration is extremely improbable for reasons outlined before. Moreover, the relatively good agreement between the observed value of $\frac{f}{f_o}$ and that calculated

from the axial ratio is direct evidence against such a large hydration. The discrepancy is certainly due to the rather wide limits of error in the original data and to deviations from the original assumptions of the Debye-Huckel theory of solutions upon which the equation is based. Solutions of 0.5 ionic strength, even of uni-univalent electrolytes, are entering the region where no known laws are obeyed and therefore it is to be expected that the results will be approximate only.

Summarizing then, the mean radius of molecularly dispersed gluten must be somewhere between 10Å and 13Å. With the present data, of a low order of accuracy, it is not possible to set closer limits.

DISCUSSION

These results serve to confirm and extend the concept of gluten as described by McCalla and Gralén (37). On the basis of ultracentrifuge and diffusion results they suggested that gluten was a reversible complex made up of molecules which varied progressively and continuously in both chemical and physical properties. On the observations of a high frictional ratio, the suggestion was made that these molecules were greatly elongated in shape but no data were presented to enable distinction between the possible alternatives of extremely high hydration of spheres or of a flattened shape. The enormous degree

of hydration necessary to explain the diffusion values given in this study on the assumption of a spherical model, clearly exclude this possibility. Moreover, Simha, by his equation applied to viscosity data, has shown that the oblate ellipsoid model is extremely unlikely for gliadin (51). Since gliadin is a derivative of gluten, it is justifiable to extend his findings to the whole complex and so we are reduced to the prolate ellipsoid as the most likely model for the gluten molecule. In sodium salicylate, these ellipsoids of revolution are capable of existing singly or in aggregation, depending on the concentration of the salt in the solution. If they are aggregated, the available evidence indicates that it is side by side association (37) rather than end to end attachment as in some viruses (48). The data given here indicate that the mean diameter of the particles making up the most soluble fraction of gluten is of the order of 25 Å and their length of the order of 400 Å to 450 Å. This applies only to the molecularly dispersed fraction, of course. Very much larger aggregates undoubtedly exist in the ordinary dispersion.

The mean surface charge density of gluten in sodium salicylate has a large uniform negative value which may be due to the reversible selective adsorption of the salicylate ions by the gluten. If this suggestion is valid it may offer a clue to the mechanism of dispersal of gluten in sodium salicylate. The bonds between salicylate ions and gluten particles may replace those between adjacent micelles, leading

to the disintegration of the gluten mass. The sol so produced would then be stabilized by the high negative charge of the particles. Unfortunately, these data give no new information on the nature of the forces between gluten molecules or gluten aggregates. It was hoped that this method might suggest an explanation of the unique properties of gluten but this hope was unfulfilled.

In addition, these data give little new light on the extent of hydration of the gluten molecule. The observations recorded here are not of sufficient precision to permit a reliable calculation of the percentage hydration. Also, it seems doubtful if the theory is sufficiently developed or the assumptions on which it is based sufficiently well fulfilled to justify the attempt at greater accuracy. In the writer's opinion, since the development of the lyophilization technique, as applied to gluten, removes many methodological barriers, a much more fruitful approach to the problem could be made by studying volume contraction. This has been used quite successfully by Neurath and Bull (39) on egg albumin.

It must be emphasized that the results given here are essentially preliminary in character. The technique and the apparatus used were not sufficiently precise to warrant more than provisional conclusions. It is hoped to confirm them by more precise observations in the future.

SUMMARY

1. Gluten dispersed in 0.5 μ sodium salicylate at 2.0°C. has been studied by means of the Tiselius electrophoretic apparatus and the Neurath diffusion cell.

2. The results of the observations indicate that gluten in this dispersing agent is electrostatically homogeneous with a high mean negative "valence" of 34 and therefore a high charge density. This charge density may be due to the selective adsorption of salicylate ions on gluten.

3. The isoelectric point of gluten in this dispersing agent is below pH 4.0.

4. The gluten particles molecularly dispersed in 0.5 μ sodium salicylate are prolate ellipsoids of revolution of the order of 25 Å in diameter and 400 to 450 Å long. When they are aggregated, it is side by side association rather than end to end.

5. The density of dehydrated gluten is 1.291 g./c.c.

6. The degree of hydration of the gluten particles is probably not great.

SUMMARY

1. Gluten dispersed in 0.5 M sodium salicylate at 5.0°C has been studied by means of the Tiselius electrophoresis apparatus and the Bauer-Pitts diffusion cell.

2. The results of the observations indicate that gluten in this dispersing agent is electrochemically homogeneous with a high mean negative "valence" or $\bar{z}$ and therefore a high charge density. This charge density may be due to the selective adsorption of salicylate ions on gluten.

3. The isoelectric point of gluten in this dispersing agent is below pH 5.0.

4. The gluten particles, molecularly dispersed in 0.5 M sodium salicylate are prolate ellipsoids of revolution of the order of 25 Å in diameter and 400 to 450 Å long. When many are superposed, it is also by side association rather than end to end.

5. The density of dehydrated gluten is 1.321 g/cc.

6. The degree of hydration of the gluten particles is probably not great.

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A P P E N D I X

APPENDIX

APPENDIX I

During the foregoing investigation, it became of interest to see if the dispersing power of sodium salicylate for gluten could be correlated with any of the structural features of the molecule or with any of the known properties of the groups attached to the benzene ring. Therefore a small experiment was set up to test the dispersing power of analogs of sodium salicylate, particularly sodium salts of organic acids. The method was as follows.

The gluten from 10 g. of ether-extracted flour was torn into small pieces and placed in 100 cc. of a .7 M solution of the reagent under test. In cases where the solubility of the substance was low, a saturated solution was used. After allowing dispersion to proceed for 3 days with occasional gentle shaking of the flask, the undispersed gluten was centrifuged off and aliquots of the supernatant dispersion were taken for nitrogen analysis. The nitrogen content of the solution was then taken as a measure of the dispersing power of the reagent at the concentration named. In cases where the reagent itself contained nitrogen, the calculated amount of nitrogen in the original solution was subtracted from the final concentration. Determinations of pH were made on all dispersions in order to estimate the effect of the H^+ concentrations. The results are listed in Table I.

APPENDIX I

TABLE I

Relative Gluten-Dispersing Powers of Reagents at .7 M

Reagent	pH	Mgm. N/c.c.
Sodium benzoate	6.8	1.66
Sodium picrate	7.5	Complete dispersion
Sodium o-methoxy benzoate	7.5	0.68
Sodium salicylate	8.4	1.41
Sodium p-nitro-benzoate	6.5	Complete dispersion
Gallic acid	7.7	1.17
Sodium sulfanilate	6.3	1.6
Sodium oleate	8.6	0.60
Sucrose	5.3	1.60
Dextrose	5.1	1.33
Maltose	5.1	1.22
Potassium iodide	6.3	0.49
Sodium potassium phthalate	7.4	.127
Benzene	5.8	.36
Water	6.3	.32
Fructose	6.0	.37
Mannite	5.3	.32
Napthol	5.3	.24
Sodium acetate	7.0	.049
Sodium citrate	7.0	.015
Sodium oxalate	6.4	.077
Sodium tartrate	6.6	.068
Potassium fluoride	7.0	.059
Phenol	4.9	.020
Phloroglucinol	5.2	.089
Pyrogallol	4.9	.076
Resorcinol	5.1	.049
Sodium acetyl salicylate	4.5	.09
Sodium formate	6.8	.05

It is evident that the majority of reagents suppressed dispersion but a few definitely encouraged it. These were, in addition to sodium salicylate itself, sodium benzoate, sodium o-methoxy benzoate, sodium picrate, sodium oleate, sucrose, dextrose, maltose, sodium p-nitro-benzoate, gallic acid and sodium sulfanilate. It seems to be significant that

TABLE I

Relative Ostwald-Dispersing Powers
of reagents at .7 M

Reagent	pH	Max. Δ\/c.c.
Sodium benzoate	6.0	1.66
Sodium picrate	7.2	Complete dispersion
Sodium o-methoxy benzoate	7.2	0.68
Sodium salicylate	6.4	1.61
Sodium p-nitro-benzoate	6.2	Complete dispersion
Gallic acid	7.2	1.17
Sodium sulfanilate	6.4	1.6
Sodium oleate	6.6	0.69
Sucrose	5.4	1.80
Dextrose	5.1	1.73
Maltose	5.1	1.22
Potassium iodide	6.4	0.89
Sodium potassium phthalate	7.6	.727
Glucose	5.8	.75
Water	6.2	.80
Fructose	6.0	.87
Mannite	6.2	.89
Heptitol	5.3	.74
Sodium acetate	7.0	.080
Sodium citrate	7.0	.010
Sodium oxalate	6.6	.077
Sodium tartrate	6.6	.066
Potassium fluoride	7.0	.059
Phenol	4.9	.020
Fluorophenol	5.6	.009
Propanol	6.2	.074
Resorcinol	5.1	.083
Sodium methyl salicylate	4.2	.09
Sodium Formate	6.8	.05

It is evident that the majority of reagents suppressed dispersion but a few definitely encouraged it. These were, in addition to sodium salicylate itself, sodium benzoate, sodium o-methoxy benzoate, sodium picrate, sodium oleate, sucrose, dextrose, maltose, sodium p-nitro-benzoate, gallic acid and sodium sulfanilate. It seems to be significant that

most of the reagents promoting dispersion have a highly polar group in close proximity to a carboxyl radical. It may be that the carboxyl and polar groups are capable of forming a ring structure involving hydrogen bonds with groups on the protein molecule such as the carboxyl and amino radicals of the constituent amino acids. Such a combination may be formed in place of similar linkages between constituent amino acids and thus may break the bonds holding molecules together. This cannot be the whole story, however, as shown by the effectiveness of sodium oleate and the sugars. It is felt that these observations justify an attempt at confirmation or extension of the data in the future.

APPENDIX II

After the removal of the molecularly dispersed fraction of gluten according to the method of McCalla and Gralén, the remaining non-gluten nitrogenous material was precipitated by dialysis of the solutions against water. This flocculent precipitate was then washed with water and redispersed in 0.5 μ sodium salicylate. Electrophoretic patterns were obtained on it as a matter of general interest. Typical patterns are shown in Fig. 1. The very small peaks are due to the low concentrations made necessary by the small amounts of material available.

These patterns show that this non-gluten material is heterogeneous in nature and probably represents a mixture of fragments. That the fragments are still relatively large is shown by their insolubility in water and their retention by the collodion membrane. Not the least important fact brought out by these patterns is that even in 0.5 μ sodium salicylate, definite components are not suppressed completely.

APPENDIX II

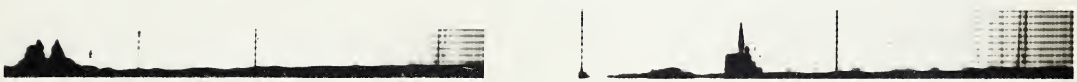

Figure 1

Electrophoresis patterns of non-gluten protein
in 0.5 μ sodium salicylate pH 5.9
Time 120' and 250'

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